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REVIEW

A framework for understanding the roles of miRNAs in animal development

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ABSTRACT

MicroRNAs (miRNAs) contribute to the progressive changes in gene expression that occur during development. The combined loss of all miRNAs results in embryonic lethality in all animals analyzed, illustrating the crucial role that miRNAs play collectively. However, although the loss of some individual miRNAs also results in severe developmental defects, the roles of many other miRNAs have been challenging to uncover. This has been mostly attributed to their proposed function as tuners of gene expression or providers of robustness. Here, we present a view of miRNAs in the context of development as a hierarchical and canalized series of gene regulatory networks. In this scheme, only a fraction of embryonic miRNAs act at the top of this hierarchy, with their loss resulting in broad developmental defects, whereas most other miRNAs are expressed with high cellular specificity and play roles at the periphery of development, affecting the terminal features of specialized cells. This view could help to shed new light on our understanding of miRNA function in development, disease and evolution.

KEY WORDS: microRNAs, Gene regulatory networks, Embryogenesis, Cell differentiation

Introduction

The regulation of gene expression lies at the heart of the cell division and diversification processes that lead to the specification of every cell type in a multicellular organism. Although transcriptional regulators are undisputed key players in directing the changes in gene expression that underlie development, post-transcriptional repressors such as microRNAs (miRNAs) have emerged as important contributors to this process. Indeed, the repression of gene expression is a recurring strategy to restrict expression patterns in time and space, thereby shaping and diversifying the gene expression profiles of different cell types during development (Hobert, 2008). In addition, repression can be utilized to maintain the level of target genes at required steady-state levels, thus providing reproducibility to processes such as development (e.g. as discussed by Cohen et al., 2006).

miRNAs constitute a diverse class of single-stranded, ∼21-24 nucleotide-long non-coding RNA molecules that are able to repress gene expression post-transcriptionally in animals and plants (Ambros, 2004; Bartel, 2009). They were initially uncovered in forward genetic screens based on their impact on development (Lee et al., 1993; Reinhart et al., 2000). All canonical miRNAs are generated from longer primary transcripts through a common mechanism that employs two sequential cleavages in order to produce the mature miRNA form (Fig. 1). Many miRNAs are produced from individual transcripts, whereas others occur in clusters such that a single primary transcript gives rise to multiple mature miRNAs. In recent years, the discovery of the highly conserved enzymes that produce mature miRNAs – Drosha and Dicer (Bernstein et al., 2001; Hutvagner et al., 2001; Lee et al., 2003) – has enabled the collective function of canonical miRNAs to be probed in various model organisms. Moreover, mature miRNAs exert their repressive function in association with a member of the Argonaute (Ago) protein family (reviewed by Iwakawa and Tomari, 2015), and the removal of this Ago effector protein has also been a useful tool for assessing the contribution of miRNA-mediated repression to different processes. For instance, in *Caenorhabditis elegans*, zygotic removal of both miRNA-dedicated Argonautes, ALG-1 and ALG-2, causes embryonic arrest during morphogenesis (Vasquez-Rifo et al., 2012). In mice, two *Ago2* null alleles lead to developmental arrest around embryonic day (E) 5.5 or 7.5 (Alisch et al., 2007; Morita et al., 2007), and loss of *Dicer1* also causes early embryonic lethality, with animals arresting before E7.5, prior to the body plan being established (Bernstein et al., 2003); however, it should be noted that Dicer is also involved in the endo siRNA pathway, so a contribution from this other class of small RNAs should be considered in the interpretation of such experiments. The deletion of *Dgcr8*, the obligate partner of Drosha, also causes embryonic lethality in mice at around day E6.5 (Wang et al., 2007).

Together, these studies highlight a need for miRNAs during embryogenesis. However, in the cases described above, maternally inherited RNAs and biogenesis/effector proteins were not eliminated, potentially masking earlier or stronger defects. Indeed, exacerbated embryonic defects have been uncovered in *Drosophila* and zebrafish following the removal of the maternal contribution of some of these factors. In zebrafish, loss of zygotic *dicer1* causes larval arrest around 10 days post-fertilization (dpf) (Wienholds et al., 2003), and maternal/zygotic mutants generated by germline transplantation undergo abnormal morphogenesis and organogenesis, and die around 5 dpf (Giraldez et al., 2005). In *Drosophila*, maternal/zygotic removal of *AGO1* results in embryonic lethality and severe deformations in both the central and peripheral nervous systems (Kataoka et al., 2001). Other attempts to remove the maternal contribution uncovered a role for miRNAs in the germline of invertebrate models. In *C. elegans*, mutants for Drosha (*drsh-1*) and Dicer (*dcr-1*) develop until adulthood owing to the maternal contribution of these factors, and only then display germline defects that lead to sterility (Denli et al., 2004; Knight and Bass, 2001). Similarly, *Drosophila* zygotic mutants for the Dgcr8 homolog, Pasha, survive until late stages in larval development but removal of maternal Pasha from the germline results in sterility (Martin et al., 2009). A temperature-sensitive allele of the gene encoding the Pasha ortholog PASH-1 in *C. elegans* also causes fully penetrant, early embryonic lethality when mothers are shifted to the non-permissive temperature around the time oocytes are produced

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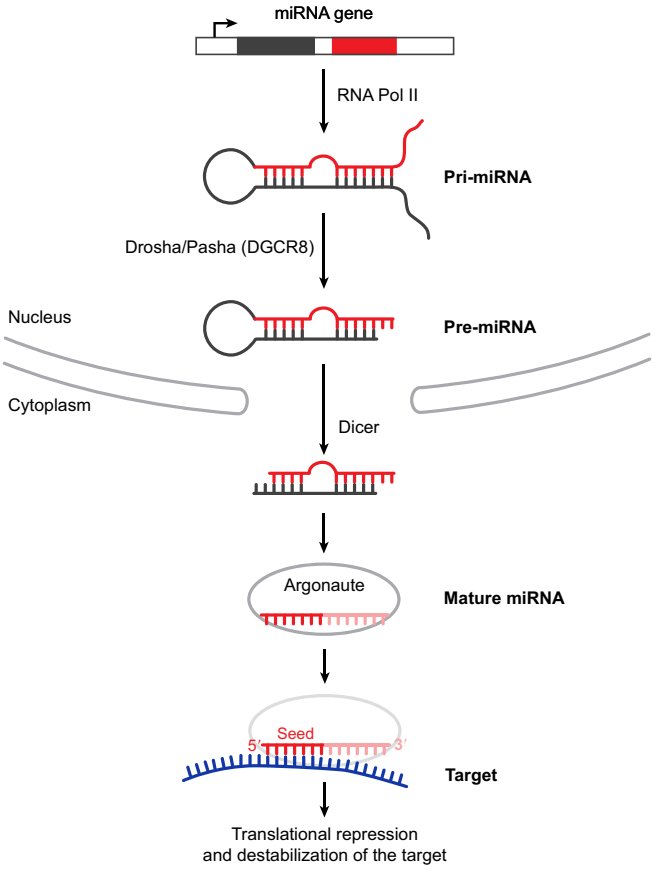

Fig. 1. An overview of miRNA biogenesis and effector pathways. The transcription of a primary miRNA (pri-miRNA) by RNA polymerase II is followed by its processing by Drosha in complex with Pasha/DGCR8, into a precursor hairpin (pre-miRNA); these steps occur in the nucleus. After nuclear export, processing of the pre-miRNA by Dicer occurs in the cytoplasm. One strand of the mature duplex is preferentially loaded onto an Argonaute family member. The miRNA then guides the Argonaute and associated factors to its targets (blue) specified by base pairing to nucleotides 2-7 at the 5′ end of the miRNA (the seed sequence, in red). For a recent review on miRNA biogenesis and its regulation, see Ha and Kim (2014).

(i.e. to eliminate the maternal contribution); by contrast, when only the zygotic component is removed, embryos are able to develop into adults, albeit sterile (Lehrbach et al., 2012). Interestingly, in mice, maternal and zygotic removal of *Dgcr8* does not cause sterility or exacerbate the defects observed in the absence of the zygotic contribution alone (Suh et al., 2010). However, maternal removal of Dicer1 and Ago2, which also act in the siRNA pathway, causes oocytes to arrest during meiosis (Kaneda et al., 2009; Murchison et al., 2007; Tang et al., 2007). This difference in outcome allowed for the discovery of the role for endo siRNAs, but not miRNAs, in mouse oogenesis (Flemr et al., 2013; Suh et al., 2010).

Although Drosha and Pasha/DGCR8 seemingly have additional non-miRNA substrates (Chong et al., 2010; Gromak et al., 2013; Kim et al., 2017; Macias et al., 2012; Rybak-Wolf et al., 2014; Triboulet et al., 2009), the fact that removal of different components that overlap with the canonical miRNA pathway causes similar effects strongly suggests that canonical miRNAs as a whole play indispensable roles during embryonic development in animals. By contrast, the deletion of many individual miRNAs seems to cause no or only subtle defects at the level of the whole organism (Chen et al., 2014; Kloosterman et al., 2007; Miska et al., 2007; Park et al., 2012). Three possible explanations for this discrepancy have been put forward and experimentally explored. Redundancy among different miRNAs is certainly one of these (as discussed in Box 1). The second possible explanation for the lack of observed phenotypes upon individual miRNA loss is that many miRNAs seem to have a modest modulatory or buffering effect on gene expression (Bartel and Chen, 2004) and act to provide robustness in the face of external or internal challenges (discussed in Box 2). And third, many miRNAs are expressed with high spatiotemporal specificity and could thus have cell-specific functions that might have been missed owing to lack of specialized assays. Although all three explanations may help us understand further how miRNAs function during development, redundancy and potential roles in

Box 1. Functional redundancies among miRNAs

Some miRNAs exist as part of families in which multiple members share the same seed sequence and, though they differ at their 3′ ends, could target the same genes, suggesting that whole families must be deleted to uncover a phenotypic consequence. This has been done systematically in *C. elegans* (Alvarez-Saavedra and Horvitz, 2010) and for specific cases in other organisms, revealing that miRNA families can act redundantly. For example, deletion of individual members of the *C. elegans mir-35*, *mir-51* and *mir-58* families has no obvious effect, but removal of all members of each family causes fully penetrant embryonic lethality in the first two cases and a variety of phenotypic abnormalities in the latter (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). Similarly, *mir-279* and *mir-996* in *Drosophila* are redundant for viability, specification of olfactory neuron subtypes and rhythmic behavior (Sun et al., 2015). Redundancies have also been uncovered among mouse miRNAs. For example, the six *mir-34*/*449* family miRNAs can be individually deleted, resulting in viable and phenotypically normal animals, whereas deletion of all members leads to high postnatal mortality, with surviving animals displaying an array of defects (Fededa et al., 2016; Song et al., 2014). In *C. elegans*, only 3/15 tested families display abnormalities, suggesting that not all miRNAs with the same seed sequence are redundant. For example, a cohort of three *let-7* homologs is expressed earlier than *let-7* and regulates an earlier larval transition (Abbott et al., 2005). Although these three miRNAs function redundantly with each other, they do not seem to be redundant with *let-7* itself. This is most likely due to their distinct spatiotemporal expression patterns, although a role for the different 3′ ends between *let-7* and its homologs has not been tested. Indeed, in zebrafish, *mir-1* and *mir-206* share the same seed sequence and are both co-expressed in muscle but seem to target different genes that affect embryonic angiogenesis differently (Lin et al., 2013), providing an example for how sequences outside the seed can be crucial for targeting specificity (Grimson et al., 2007). Similarly, *mir-790* and *mir-791* in *C. elegans* are co-expressed in CO_2-sensing neurons and share the same seed sequence, but only *mir-791* has a functional role in CO_2 sensing (Drexel et al., 2016).

Although potential redundancies between miRNAs of similar sequence are easy to identify, functional redundancies with other genes also exist. These can be between two miRNAs that co-target the same mRNA, or between a miRNA and another gene (miRNA or not) affecting the same process through different target genes. For example, in *Drosophila*, whereas single mutants for *mir-1* and *mir-9a* do not show obvious embryonic problems, double mutant embryos show early lethality caused by defects during ventral furrow formation (Fu et al., 2014). These two miRNAs are unlikely to target the same gene(s) as they are expressed in different germ layers. Other cases of miRNAs acting within different genetic pathways can be inferred from a study in which several *C. elegans* miRNA mutants that display no defects on their own, exhibit phenotypes in different sensitized genetic backgrounds (Brenner et al., 2010). A case of two different miRNAs targeting the same mRNA has been recently reported in *C. elegans*: *mir-35* and *mir-58* family miRNAs cooperate to keep the trigger of apoptosis, EGL-1, below a certain threshold, preventing precocious cell death during embryonic development (Sherrard et al., 2017).

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Box 2. The roles of miRNAs in providing developmental robustness

Development is remarkably robust to perturbations, both external (i.e. changes in the environment) and internal (i.e. variations in the genotype or the inherent noise associated with gene transcription). This is at least in part due to the robustness of the GRNs that drive development and, within these, some miRNAs have an important contribution. For example, *Drosophila* lacking *mir-7* develop normally in standard laboratory conditions, but if larvae are grown under fluctuating temperature, they develop defects in sensory neuron differentiation (Li et al., 2009). In *C. elegans*, *mir-34;mir-83* double mutants have a low penetrance defect in gonad morphogenesis that is significantly enhanced if animals are subjected to temperature oscillations (Burke et al., 2015). More recently, *mir-139* and *mir-24* have been shown to be required for robust vasculature development in zebrafish (Kasper et al., 2017). This was evidenced not only in the increased sensitivity of animals lacking these miRNAs to diverse stressors, but also in the increase in variance of some of the observed traits, even under standard conditions. Such an increase in phenotypic variance has been observed for other miRNA mutants in *Drosophila* and provides an indication that these miRNAs are necessary to buffer variability in gene expression (e.g. Cassidy et al., 2013; Kugler et al., 2013). The fact that some miRNAs play roles in providing robustness to development and other biological processes can also explain why some miRNA functions have been challenging to uncover in the laboratory.

Two properties of miRNA-mediated regulation make miRNAs good candidates for providing robustness (discussed further by Posadas and Carthew, 2014). First, the relatively modest repression exerted by some miRNAs can be utilized as a weak buffer for the inherent 'noise' of transcription or to set a threshold of target activity (Cohen et al., 2006; Li et al., 2006). Second, the function of miRNAs within extensive feedback and feedforward regulatory loops allows these GRNs to adapt to gene expression fluctuations and return the level of crucial components to the set steady state (Posadas and Carthew, 2014).

robustness are the concepts that have been considered most extensively (e.g. Alvarez-Saavedra and Horvitz, 2010; Posadas and Carthew, 2014). However, as more functions of miRNAs are being uncovered, it is now possible to begin to distinguish between the contributions of cell type-specific miRNAs and those that act more globally.

Here, we discuss miRNA expression and function in the context of a framework based on the hierarchical nature of developmental processes. In this view, a relatively small fraction of miRNAs acts at the top of the hierarchy, displaying essential functions early during embryogenesis, whereas many other miRNAs seem to act at lower levels of the hierarchy, with a number of miRNAs playing roles in the final stages of development, providing specific cell types with specialized properties. Although we focus on miRNAs that act during development, we note that many other miRNAs certainly function in cellular homeostasis or other processes during adult life or aging; these miRNAs will not be covered here.

Two main classes of miRNAs can be identified during animal development

The first miRNAs that were discovered can be generally classified in two broad groups. In the first, we find miRNAs such as the founding members, *lin-4* and *let-7*, which were identified in screens for genes involved in developmental timing in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000), and loss of which dramatically impairs progression through larval development. Also in this group are *Drosophila bantam* and *mir-14*, loss of which results in lethality as well as small body size (Brennecke et al., 2003; Hipfner et al., 2002; Xu et al., 2003). These miRNAs are generally broadly expressed in many, if not most, tissues, and affect essential cellular behaviors such as the decision to divide, differentiate or die. Their loss thus results in easily observable mutant phenotypes. In the second group, we find miRNAs such as *C. elegans lsy-6*, which is expressed in a single sensory neuron in the worm (Cochella and Hobert, 2012a), is essential for the specification of that particular neuron, and does not have additional functions in the animal (Johnston and Hobert, 2003). Similarly, *Drosophila mir-279* and mouse *mir-96*, also uncovered in forward genetic screens, have been implicated in very specific sensory contexts, namely CO_2 sensing and hearing, respectively (Cayirlioglu et al., 2008; Lewis et al., 2009).

How can these two groupings help us understand the way miRNAs contribute to development? Eric Davidson pioneered the view that development can be explained by the complete map of interactions between regulatory genes that eventually define when and where different biochemical and structural functions happen within the animal (Davidson and Erwin, 2006; Davidson et al., 2003). These gene regulatory networks (GRNs) are hierarchical, with some components regulating the initial morphological patterning events at the top or core of the hierarchy, and others promoting the detailed functions of cell differentiation at the bottom or periphery of the hierarchy (Fig. 2). GRNs are also canalized, meaning that the establishment of a given regulatory state in a region of the embryo restricts subsequent processes, such that the gene regulatory events that follow take place exclusively within that domain. In this context, it is evident that the effect of any regulatory factor – a miRNA or any other type of regulator – on the organism strongly depends on its position in the hierarchy of the networks that control developmental gene expression. The loss of miRNAs acting in the core of the hierarchy can be expected to have more broad consequences at the organismal level than loss of miRNAs acting at the periphery, which might have a crucial effect on specific cell types but have only subtle impact on the organism. Of course, certain specific cell types are essential for organismal function and viability and their failure to develop properly can still result in lethality. Therefore, it is possible that miRNAs, or other regulators, acting at the periphery have essential functions, although these will likely manifest at later stages of embryogenesis or even postnatally, e.g. *mir-451* in red blood cells (Cheloufi et al., 2010; Patrick et al., 2010).

Placing miRNAs in such a context has two main implications. First, the realization that many miRNAs may act exclusively at the periphery of development, in the final stages of cellular differentiation, could explain why deletion of at least some of these miRNAs seemingly causes no or subtle defects (Chen et al., 2014; Kloosterman et al., 2007; Miska et al., 2007; Park et al., 2012). Regardless of whether a cell-specific miRNA acts to buffer gene expression under stressful conditions or to strongly repress its target and define the fate of a cell (e.g. *C. elegans lsy-6*), the function of such a miRNA can only be revealed with specific cell-fate markers and functional assays (e.g. Chang et al., 2004; Johnston and Hobert, 2003). This view suggests that other miRNAs in this group might have functions that have been missed owing to the lack of appropriate assays, and allows for better hypotheses to be formulated when studying the function of such miRNAs. Second, identifying miRNAs that act at similar levels in the hierarchy of development can allow for the identification of redundancies and synergisms among different miRNAs. Indeed, a substantial amount of functional redundancy exists between different miRNAs and between miRNAs and other components of the GRNs they are a part of (see Box 1). Thus, placing miRNAs in this framework can help guide the exploration of further redundancies to uncover the contributions of other miRNAs to development.

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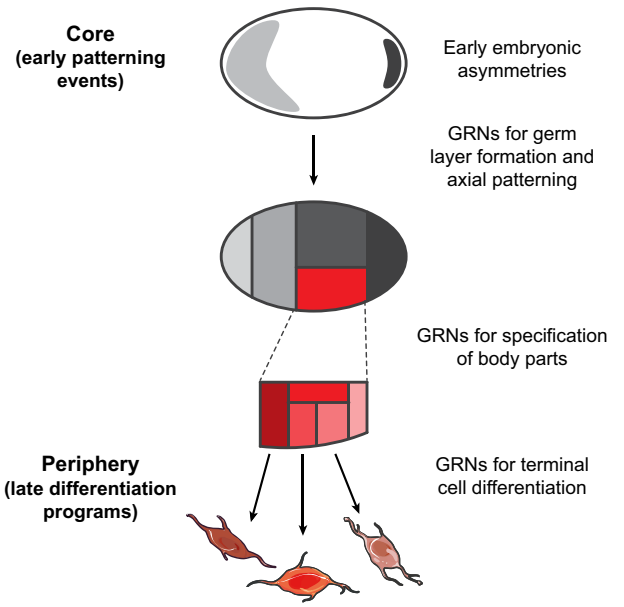

Fig. 2. Hierarchical organization of the gene regulatory networks that control embryonic development. At the onset of embryogenesis, gene regulatory events are triggered by asymmetries in the oocyte and sperm that result in regionalization of maternal and eventually early zygotic regulatory factors. These represent the input for new gene regulatory networks (GRNs) that progressively narrow down the potential identities of cells until the point of terminal differentiation. 'Core' events are those required to set up the basis for embryogenesis and place the appropriate progenitors in the right place at the right time. Further specification of those progenitors and differentiation into different specialized cell types is driven by GRNs at the 'periphery'. Based on Peter and Davidson (2011).

Many miRNAs may act at the periphery of development

The classification presented above implies a correlation between the function and the expression pattern of a given miRNA. Genes at the top of the hierarchy should be present earlier in development and, as seen in the cases presented below, tend to be broadly and abundantly expressed. Conversely, miRNAs acting exclusively at the periphery are likely to be expressed at later time points in development and in a cell type-restricted manner. It is known that many miRNAs are expressed with specific spatiotemporal patterns (Aboobaker et al., 2005; Landgraf et al., 2007; Martinez et al., 2008; Park et al., 2012; Wienholds et al., 2005). However, to gain insight into what fraction of miRNAs might play roles exclusively at the periphery, in specific cell populations, a complete view of the expression of all miRNAs throughout the development of all cell types is needed. Despite the expression analyses mentioned above, this level of completeness has not yet been achieved. However, a good approximation can be obtained by examining miRNA sequencing data from whole animals at different developmental stages and complementing this with expression data of individual miRNAs. For example, sequencing data from whole embryos or larvae of *C. elegans* and zebrafish (Kato et al., 2009; Yao et al., 2014) show that a small fraction of miRNAs is highly abundant during at least one developmental stage, whereas the majority seems to be expressed at low levels (Fig. 3). Among the latter, some miRNAs may be broadly expressed at low levels in each cell where they are present, which makes it unlikely that they will have measurable functions (see review by Ameres and Zamore, 2013). On the other hand, other seemingly lowly expressed miRNAs might be present at high concentrations, albeit within restricted cell types, and could therefore represent peripheral miRNAs.

Expression analysis of miRNAs that fall into the 'low expression' category in *C. elegans* shows that several of these exhibit very specific expression patterns (Martinez et al., 2008; and transcriptional reporters generated in our lab; Fig. 3A). This group contains, among others, miRNAs such as *lsy-6*, which is expressed from embryogenesis through to adulthood in a single neuron (Cochella and Hobert, 2012a), as well as *mir-791*, which is expressed in three pairs of neurons throughout development (Drexel et al., 2016). This view suggests that as many as three out of four *C. elegans* miRNAs could be expressed in relatively few cells and thus be restricted to playing cell type-specific roles in development. Conversely, among the most abundant miRNAs in the *C. elegans* embryo are the *mir-35* and *mir-51* families, which together account for ∼75% of all embryonic miRNAs (Fig. 3A). Consistent with them displaying a core position in the GRN, these are the only two miRNA families known to be necessary for the development of viable *C. elegans* embryos (Alvarez-Saavedra and Horvitz, 2010).

A similar distribution can be observed for miRNAs in zebrafish embryos, with a group of highly expressed miRNAs and a larger fraction of seemingly lowly expressed ones (Fig. 3B). Several *in situ* hybridization studies (e.g. from Wienholds et al., 2005) support the hypothesis that several of the lower abundance miRNAs are actually expressed in highly restricted cell populations (some are shown in Fig. 3B), although we cannot exclude the possibility that some of these miRNAs are highly and broadly expressed only at later stages.

Several miRNAs occupy intermediate levels in the developmental hierarchy (Fig. 2) by acting in broader tissues or organs, and can thus impact a significant part of the organism without acting in early patterning events. For example, *mir-1*, *mir-9* and *mir-124* are necessary for broader muscle cell and neuronal differentiation in vertebrates (Sokol, 2012; Cochella and Hobert, 2012b). Moreover, it should be noted that the positions of miRNAs within the hierarchy need not be exclusive: some miRNAs that occupy core positions can also play additional roles at the periphery, in the same way that many transcriptional regulators and signaling pathways that are used for early patterning events in embryogenesis are re-employed later in development in specific contexts of cell differentiation. Below, we focus primarily on miRNAs acting at the core of embryonic GRNs and those acting in the periphery, in each case highlighting the key cell and developmental processes that are regulated by these miRNAs.

Core developmental processes in which miRNAs are involved

The production of an embryo begins with fertilization of an oocyte, which contains high amounts of RNAs and proteins produced by the mother during oogenesis. In many species, the asymmetric localization or functional segregation of some of these components is essential to pattern the embryo (Farley and Ryder, 2008). As embryogenesis then progresses, these maternal components are cleared to give way to zygotic gene expression, and this is a process in which miRNAs have been implicated. In addition, miRNAs have been shown to regulate several cellular processes (Fig. 4) including cell division, intercellular signaling, differentiation and apoptosis, all of which are essential to coordinate growth and pattern formation during embryonic development. In general, these miRNAs that regulate core developmental process are also those that display the most conservation, although there are some exceptions (see Box 3).

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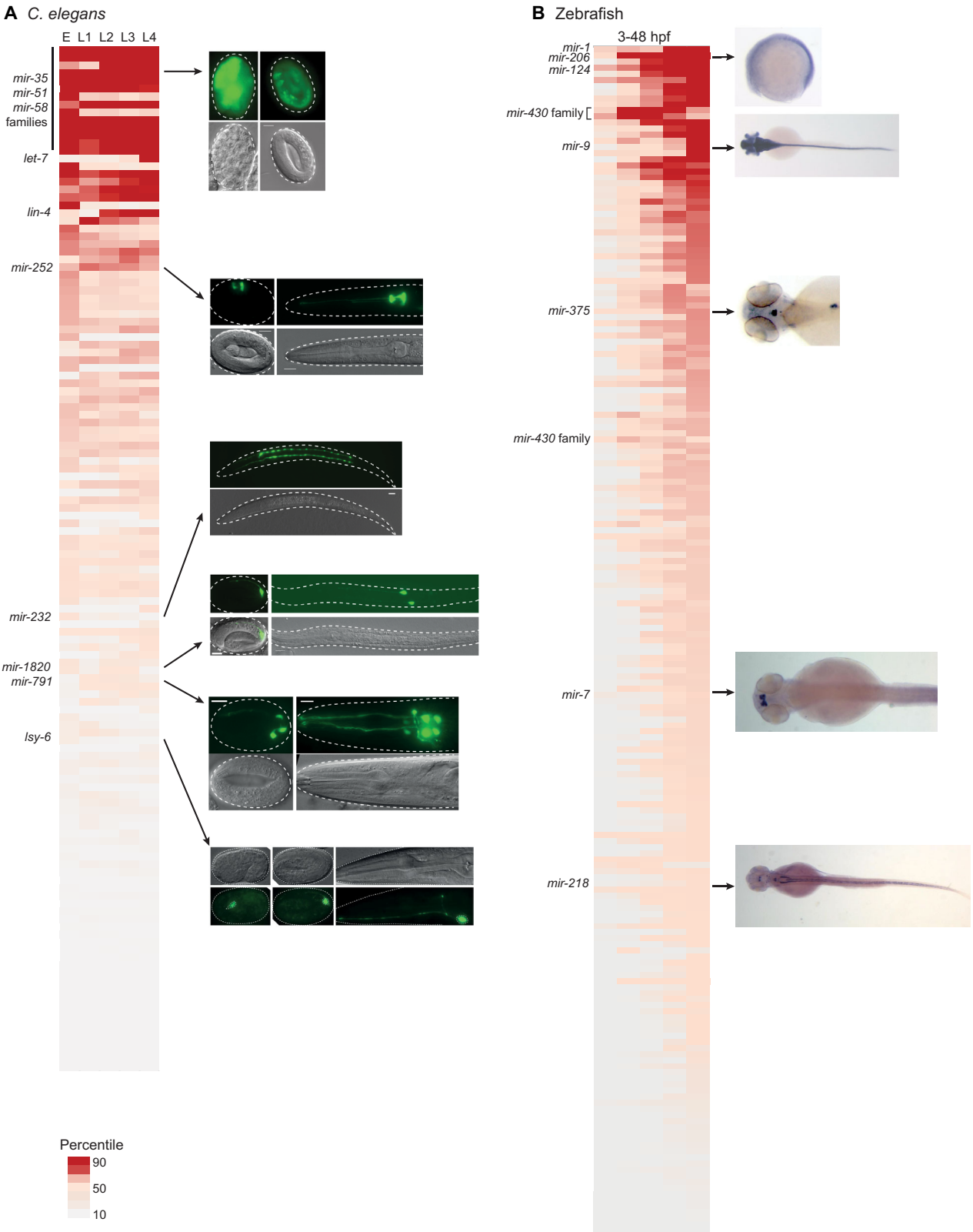

Fig. 3. The abundance and expression of miRNAs. (A) *C. elegans* miRNA sequencing data ranked by the maximal expression at any developmental stage. miRNAs were sequenced from whole embryos (E) or larvae (L1 to L4). Data are replotted from Kato et al. (2009). The miRNAs mentioned in the main text are highlighted and the expression patterns of some of these (based on transcriptional reporters) is shown to the right to highlight the correlation between expression level and pattern. The high abundance of the *mir-35* cluster, for example, correlates with its broad expression. Several seemingly low abundance miRNAs, such as *lsy-6* and *mir-791*, are expressed in a highly restricted manner. The *lsy-6* expression pattern is reproduced with permission, from Cochella and Hobert, 2012a. The *mir-791* expression pattern is reproduced from Drexel et al., 2016. (B) miRNA sequencing data from zebrafish embryos ranked as for *C. elegans*. Data are replotted from Yao et al. (2014). Expression patterns of selected miRNAs (as detected by *in situ* hybridization) are reproduced with permission from Wienholds et al. (2005). hpf, hours post-fertilization.

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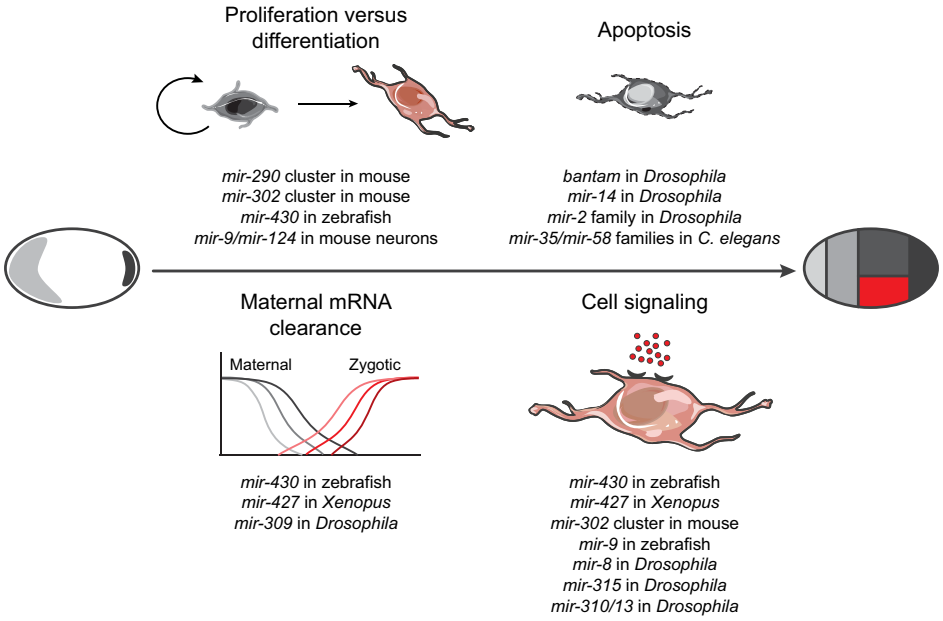

Fig. 4. Core cell and developmental processes that are regulated by miRNAs. Summary of the key cellular and molecular processes required for early events in embryogenesis in which miRNAs have been implicated. Examples of miRNAs that regulate these processes in various organisms are shown.

Maternal mRNA clearance

A number of miRNAs play a role in clearing maternal mRNAs during early embryogenesis. In zebrafish, *mir-430* is encoded by multiple genomic copies, allowing for robust production of this miRNA at the onset of zygotic genome activation (Giraldez et al., 2005). This has precluded the generation of a *mir-430* null animal so far. However, zebrafish embryos lacking maternal and zygotic Dicer1 accumulate hundreds of maternal transcripts that are enriched for *mir-430* binding sites, and the re-introduction of mature *mir-430* into these embryos substantially suppresses this maternal mRNA accumulation by promoting deadenylation and thus destabilization of *mir-430* targets (Giraldez et al., 2006). The ortholog of *mir-430* in *Xenopus laevis*, *mir-427*, has also been shown to trigger deadenylation of maternal mRNAs during the maternal-to-zygotic transition in frog embryos (Lund et al., 2009). An unrelated miRNA family in *Drosophila*, the *mir-309* cluster, is also highly expressed early during zygotic genome activation, and its loss results in maternal mRNA accumulation (Bushati et al., 2008). However, the impact of the maternal mRNA clearance function of *mir-430* and *mir-309* on embryogenesis remains unclear. In the case of *mir-309*, this is because the bulk of maternal mRNA clearance in the fly embryo is mediated by the RNA-binding protein Smaug (Tadros et al., 2007). In the case of *mir-430*, it has been shown that maternal/zygotic Dicer-deficient zebrafish embryos still manage to activate the zygotic program despite the strong delay in maternal mRNA clearance (Giraldez et al., 2006). In addition, *mir-430* has other independent functions that could explain the morphogenesis defects observed in Dicer-deficient embryos (discussed below).

Cell proliferation

Several miRNAs appear to play a role in controlling proliferation. For instance, members of the *mir-290* cluster, which contains the most highly expressed miRNAs in mouse embryonic stem cells (ESCs), have been proposed to act redundantly to promote G1-S transition and thus proliferation in ESCs. These miRNAs are able to repress the cell cycle inhibitor gene *Cdkn1a* as well as two other key regulators of the G1-S transition (Wang et al., 2008). However, deletion of the *mir-290* cluster in mouse embryos results in only partially penetrant lethality in mid-late embryogenesis, and this is accompanied by a reduction in germ cells that does not seem to be due to cell cycle arrest or increased apoptosis (Medeiros, 2011). Whether the cause of embryonic lethality in these animals is directly related to cell proliferation defects in other cell types remains to be tested. Interestingly, a recent study has reported functional redundancy between the *mir-290* cluster and the *mir-302* cluster (Parchem et al., 2015), members of which share the same seed sequence as the *mir-290* family and have overlapping expression patterns in the mouse embryo between E5.5 and E6.5 (Parchem et al., 2014). The specific knockout of *mir-302* results in mutant embryos that are grossly abnormal by E9.5, exhibiting large open anterior neural tubes and severely affected brain development, with no recoverable mutants at E18.5 (Parchem et al., 2015). Deletion of both the *mir-290* and *mir-302* clusters results in fully penetrant, early synthetic lethality around the time of gastrulation. The cellular and molecular effects of these combined deletions will be interesting to explore further, as this is the first report of an absolute requirement for specific miRNAs early during mammalian development. In addition, the comparison of these miRNA-mutant embryos with those lacking the miRNA biogenesis/effector machinery and further tests could determine whether the *mir-290/mir-302* family accounts for the essential requirement for those proteins (Alisch et al., 2007; Bernstein et al., 2003; Wang et al., 2007).

Interestingly, the *mir-290/mir-302* family is orthologous to zebrafish *mir-430*. However, the potential role of this miRNA family in maternal mRNA clearance has not been explored. Like mouse *mir-302*, zebrafish *mir-430* is required for neural tube formation, and its loss – like the loss of mouse *mir*-302 – results in defects in neural tube morphogenesis. Whereas in the mouse the observed malformation was attributed to premature differentiation of the neuroectoderm leading to an over-proliferation defect (Parchem et al., 2015), in zebrafish it is proposed to be due to abnormal spindle orientation during the division of progenitors (Takacs and Giraldez, 2016). However, such studies are difficult to compare as they both measure and describe different parameters of the observed phenotypes, and it will thus be important to explore further whether a unifying mechanism for this miRNA family exists across species.

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Box 3. The evolution and conservation of 'core' miRNAs

It may be expected that broad and abundant miRNAs involved in core developmental process are also among the most conserved animal miRNAs (Chen and Rajewsky, 2007). Indeed, this seems to be the case for many of them, e.g. the *mir-51* family required for embryogenesis in *C. elegans* is a homolog of the most ancient animal miRNA, *mir-100* (Grimson et al., 2008); *let-7* is also highly conserved and plays broad and essential roles in many contexts (Mondol and Pasquinelli, 2012); and the *mir-430*/*302*/*290* family also seems to have maintained a core position in development during vertebrate evolution (Giraldez et al., 2005; Parchem et al., 2015; Rosa et al., 2009). However, the *mir-35* family, which is essential for *C. elegans* embryonic development, seems to be nematode specific. Interestingly, recent studies show that the *mir-35* family acts in the sex determination pathway in *C. elegans* (McJunkin and Ambros, 2014, 2017). Sex determination pathways have evolved independently in different animals, suggesting that miRNAs could have become essential also at later points in evolution as they were co-opted for species-specific essential processes. Interestingly, other small RNAs have been implicated in sex determination, such as a Piwi-interacting RNA (piRNA) in the silkworm (Kiuchi et al., 2014) and the miRNA *let-7* in *Drosophila* (Fagegaltier et al., 2014), pointing to another potential link between the rapid evolution of certain biological processes and the cooption of evolutionarily fluid mechanisms such as small RNA-mediated repression (Shi et al., 2013).

Other miRNAs that have been shown to play a role in proliferation, influencing the decision to divide or differentiate, are mouse *mir-9* and *mir-124*. Both are neuronal miRNAs highly expressed in the developing mouse brain during early embryogenesis, although in more restricted cell populations, starting from E9-E10.5 and persisting after birth (Maiorano and Mallamaci, 2009; Shibata et al., 2008). In vertebrates, they are crucial to orchestrate successfully the transition between progenitors and differentiated neurons through the regulation of multiple targets that either promote progenitor proliferation or directly inhibit neuronal differentiation, most of which are transcriptional regulators (Cheng et al., 2009; Maiorano and Mallamaci, 2009; Shibata et al., 2008). Interestingly, *mir-9* and *mir-124* play an instructive role during the *in vitro* differentiation of human fibroblasts into neurons, acting synergistically with neurogenic transcription factors (Yoo et al., 2011).

Apoptosis

A number of miRNAs have been implicated in the regulation of programmed cell death events during development. For example, *Drosophila bantam* was identified in gain-of-function screens for genes that are important for the regulation of tissue growth rates. Bantam is highly expressed during embryogenesis, and its overexpression causes profound tissue overgrowth whereas deletions lead to reduced larval growth, pupal lethality and loss of imaginal discs (Brennecke et al., 2003; Hipfner et al., 2002). The dissection of *bantam* function revealed that it has genetically distinguishable death-inhibiting and growth-promoting activities: the first is due to its ability to directly suppress the pro-apoptotic gene *hid* (Brennecke et al., 2003), whereas the second, pro-proliferative, function has been related to inhibition of basal ecdysone production, which negatively affects tissue growth through repression of Myc (Boulan et al., 2013). Another *Drosophila* miRNA that has been implicated in controlling cell death is *mir-14*, which was identified due to the ability of *mir-14* null mutants to enhance a pro-apoptotic phenotype. These mutants die during pupal development and also show increased levels of acylglycerols (Xu et al., 2003). Many, though not all, of the defects observed in *mir-14* mutants seem to be due to loss of repression of the ecdysone receptor in these mutants (Varghese and Cohen, 2007). In fact, the programmed death of the salivary glands during metamorphosis requires *mir-14*-mediated induction of autophagy through repression of an inositol trisphosphate kinase, IP3K2 (Nelson et al., 2014). Members of the *Drosophila mir-2* family have also been proposed as regulators of pro-apoptotic genes, including *reaper* (*rpr*), *grim* and *sickle* (*skl*) (Stark et al., 2003). The combined loss of two *mir-2* family members, *mir-6* and *mir-11*, causes strong embryonic lethality, with mutants exhibiting a strong defect in the organization of the embryonic central nervous system (Ge et al., 2011). Pro-apoptotic genes were upregulated by 2- to 4-fold in these mutants, suggesting that the observed defects were due to elevated apoptosis. In support of this, removal of one copy of each of *rpr*, *skl* and *hid* strongly suppressed the lethality and restored the normal central nervous system patterning in mutant embryos.

A number of *C. elegans* miRNAs have also been identified as cell death regulators. During *C. elegans* development, programmed cell death occurs in a highly reproducible manner such that 131 cells are invariantly eliminated, 113 of them during embryogenesis (Sulston et al., 1983). Apoptosis is triggered by expression of a BH3-only gene, *egl-1*, which is under tight transcriptional control (Nehme and Conradt, 2008). A recent study showed that *egl-1* is already transcribed in the mother of cells destined to die, but two miRNA families, the highly expressed *mir-35* and *mir-58* family miRNAs, co-target *egl-1* to maintain its dose below a certain threshold. Accordingly, loss of these miRNAs results in premature death of those cells in which *egl-1* is de-repressed above that threshold (Sherrard et al., 2017). Interestingly, the *mir-58* family shares sequence homology with *Drosophila bantam*, suggesting functional conservation, even though their targets are not related.

Cell-cell communication

Cell adhesion and intercellular signaling are essential to instruct spatial domains within the early embryo to assume different regulatory states and accomplish the correct patterning of the body plan. A number of miRNAs have been shown to regulate signaling pathways during embryogenesis, albeit at different positions in the hierarchy of development as defined by Davidson (Peter and Davidson, 2011). The signaling pathway regulated by miRNAs that is placed highest in this hierarchy during vertebrate embryogenesis is the Nodal pathway, which is crucial for germ layer formation (Feldman et al., 1998; Zhou et al., 1993). Nodal signaling is regulated by the *mir-430/mir-290/mir-302* family in zebrafish, *Xenopus*, mouse and human ESCs. In zebrafish and *Xenopus*, both agonists and antagonists of the Nodal pathway are reported to be repressed by these miRNAs, suggesting a requirement for achieving the right balance in signaling through this pathway (Choi et al., 2007; Rosa et al., 2009). In human ESCs, the two antagonist Lefty genes, but not *NODAL*, seem to be targeted by *mir-302* (Rosa et al., 2009). It will be interesting to test how removing *mir-290* and *mir-302* affects Nodal signaling in the mouse.

Other miRNAs have also been implicated in modulating different signaling pathways in a variety of model systems, at different points in development and, in some cases, in multiple cellular contexts. Examples are the regulation of fibroblast growth factor signaling by *mir-9* in zebrafish (Leucht et al., 2008), or of the Wnt/β-catenin pathway by *mir-8*, *mir-315* and *mir-310/13* in *Drosophila* (Kennell et al., 2008; Pancratov et al., 2013; Silver et al., 2007). These and other examples have been more extensively reviewed elsewhere (Hagen and Lai, 2008; Luhur et al., 2013).

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miRNAs with roles in the periphery of development

Although a number of miRNAs, as discussed above, have acquired essential functions for embryonic development, many others have not. Based mostly on expression profiles, but also on the observation that deletion of many individual miRNAs does not cause broad defects during early development, it seems likely that a fraction of miRNAs is instead restricted to playing roles at later stages of development, for example during the differentiation and specialization of specific cell types. Some examples of such miRNAs with restricted expression patterns and specialized functions do exist in different animals, as we highlight below.

Diversification of neuronal development and function

Nervous systems are composed of a large cellular diversity, with numerous neuronal and glial cell types. Several miRNAs have been implicated in either the specification of particular neuron types or in the acquisition of specialized traits for their optimal function. Here, we describe a few of these (summarized in Fig. 5).

A cell fate switch

Restriction of miRNA expression and function is particularly extreme in the case of *C. elegans*, as cellular classes can be represented by as little as a single cell. Such is the case for a class of gustatory neurons represented by a bilateral pair of neurons – the ASE left (ASEL) and ASE right (ASER) neurons. Each member of the pair expresses a distinct terminal gene battery and thus adopts a different functional identity (Hobert, 2014). Importantly, it was shown that the miRNA *lsy-6* is expressed exclusively in the left member of this pair (Fig. 5A) and, through repression of a transcription factor called COG-1, is necessary for adoption of the left neuron-specific identity. In line with this, it was shown that the ectopic expression of *lsy-6* in the neuron on the right side is sufficient to convert the identity of this cell into a left-like identity (Johnston and Hobert, 2003). Therefore, *lsy-6* acts as a cell-fate switch between two neuronal identities.

A repressor of broadly expressed genes

Like *lsy-6*, the *C. elegans* miRNA *mir-791* is expressed at low levels in whole embryos or larvae (Fig. 3A; Fig. 5B) and was recently shown to be exclusively expressed in three pairs of chemosensory neurons in the head of the worm (Drexel et al., 2016). These neurons were known to be involved in CO_2 sensing, and this led to the finding that *mir-791* is required in these neurons for the correct avoidance response of *C. elegans* to high CO_2 concentration. The targets of this miRNA are, surprisingly, two practically ubiquitous genes that are specifically repressed in the *mir-791*-expressing neurons, showing that cell-specific repression of otherwise broadly expressed genes is necessary for the correct function of specialized cells (Drexel et al., 2016). Post-transcriptional regulation, for example by a miRNA, may be an advantageous mechanism to differentially regulate gene expression when transcriptional regulation is constrained. This seems to be the case for broadly expressed genes, for which the promoter-enhancers are compact and are likely more difficult to be regulated at the transcriptional level than the modular, developmentally regulated enhancers (Zabidi et al., 2015).

A homeotic regulator of neuronal function

The *Drosophila* Bithorax complex (BX-C) Hox cluster contains three homeobox genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), as well as a bidirectionally transcribed miRNA locus (Fig. 5C). This locus, which lies between the *abd-A*

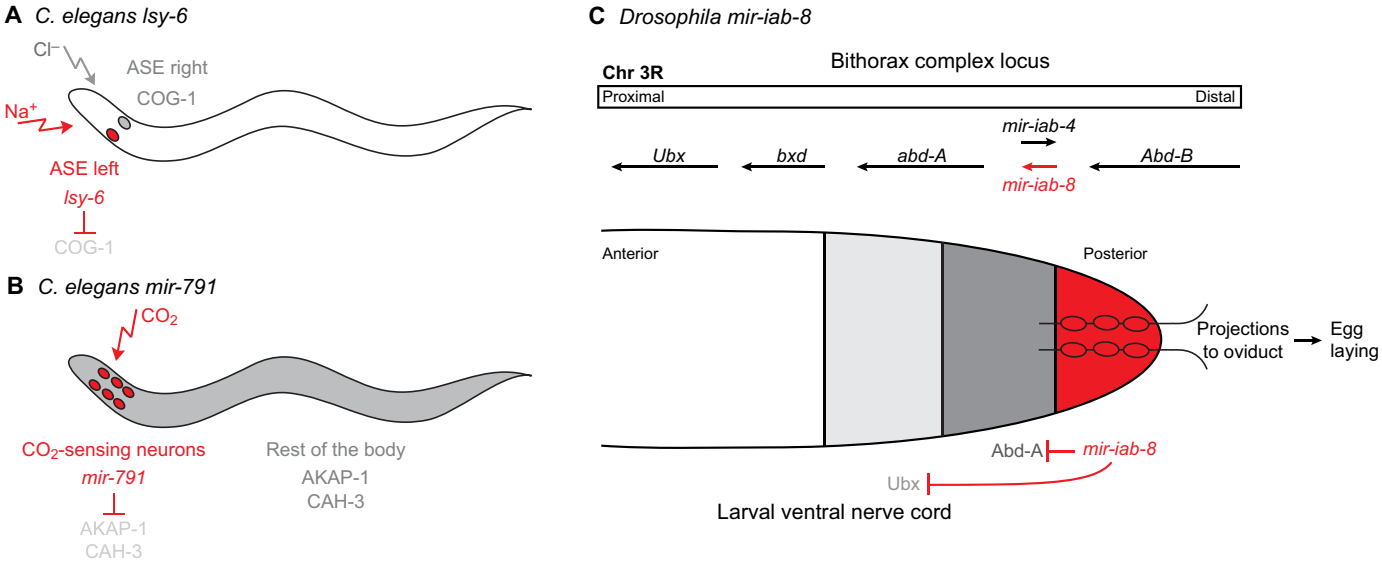

Fig. 5. Examples of miRNAs that perform cell-specific or cell-restricted functions. miRNAs specifically expressed in a restricted domain of an organism are very likely to perform tissue- or cell type-related functions. Three known examples of miRNAs operating in such narrow cellular contexts are represented here. (A) In *C. elegans*, *lsy-6* is exclusively expressed in the ASE left neuron (ASEL), where it represses the transcription factor COG-1, allowing the neuron to adopt an ASEL fate; by contrast, the ASER neuron expresses COG-1. This molecular asymmetry translates into a functional asymmetry in the ability of both neurons to sense distinct water-soluble cues. (B) In the head of *C. elegans*, the expression of *mir-791* is restricted to the three main CO_2-sensing neuronal pairs, in which *mir-791* represses two otherwise ubiquitously expressed genes (those encoding AKAP-1 and CAH-3). This strategy allows the function of these specialized neurons to be optimized for orchestrating the avoidance response of the worm to high CO_2 concentrations. In both of these examples (*lsy-6* and *mir-791*), the neurons are born and specified during the late stages of embryogenesis, the time at which both miRNAs are expressed. (C) *mir-iab-8* is a miRNA transcribed from a bidirectional miRNA locus that is part of the broader *Drosophila* Bithorax complex Hox cluster. The expression of *mir-iab-8* follows Hox cluster co-linearity, and *mir-iab-8* localizes to a posterior segment of the larval ventral nerve cord, where it is crucial for the repression of two Hox genes that play a role in the correct generation and development of motor neurons that innervate the oviduct and trigger egg laying.

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and *Abd-B* genes, produces two miRNA hairpins: *mir-iab-4 and mir-iab-8*. Flies lacking this miRNA locus are sterile, and a more precise dissection of the locus showed that this was primarily due to loss of *miR-iab-8* (Bender, 2008). More specifically, it was shown that *mir-iab-8* expression follows Hox cluster co-linearity and that the miRNA is present in a band in the posterior end of the embryo and later becomes restricted to a posterior segment of the larval ventral nerve cord (VNC). This region of the VNC gives rise to, among others, motor neurons that innervate the oviduct and are essential for egg laying. Under normal conditions, *mir-iab-8* represses two Hox transcription factors. However, in its absence their de-repression results in the generation of motor neurons that fail to innervate the oviduct sufficiently, explaining the observed sterility in the absence of *mir-iab-8* (Garaulet et al., 2014).

Other lineage-specific miRNAs in vertebrates

The analysis of miRNAs that exhibit restricted expression has also led to the identification of a large number of miRNAs that display lineage-restricted expression patterns and appear to function in a cell type-restricted manner. These include miRNAs in specific neurons and glia (Cochella and Hobert, 2012b), in certain cells of the heart (Small and Olson, 2011), and those present during cartilage and bone development (Papaioannou et al., 2014), among others.

For instance, *mir-375* is involved in pancreatic islet development and function in zebrafish and mice. This miRNA is abundant in murine pancreatic islets and pancreatic cell lines but is not expressed in other tested tissues (Poy et al., 2004), and in zebrafish is expressed exclusively in pancreatic islets and the pituitary gland (Wienholds et al., 2005). The morpholino-mediated loss of *mir-375* function in fish showed that it is required for pancreatic islet structure, acting through unknown targets, although islet cells still seem to express known markers and produce insulin (Kloosterman et al., 2007). In pancreatic cell lines, a deficit in insulin production was observed upon *mir-375* inhibition with a siRNA (Poy et al., 2004). Further studies *in vivo* are thus needed to help understand the contribution of this miRNA to pancreas development and function.

The murine hematopoietic lineage has also provided a fertile ground for finding cell type-specific miRNAs. For example, *mir-150* exerts crucial regulation as cells transition from the pro-B to the pre-B stage during mature B-cell production, acting via repression of the transcription factor Myb (Xiao et al., 2007; Zhou et al., 2007). Consistently, *mir-150*$^{-/-}$ mice are viable and morphologically normal, but show specific expansion of the B lineage (Xiao et al., 2007). In the myeloid lineage, *mir-223* is expressed during the differentiation of granulocytes and appears to act through repression of a key transcription factor for myeloid development, Mef2c, to regulate granulocyte production (Johnnidis et al., 2008). Thus, although *mir-223* mutant mice are born at normal ratios and are viable and fertile, they have increased numbers of granulocyte progenitors and circulating neutrophils (Johnnidis et al., 2008).

Conclusions

Over the last decade, the specific functions of numerous miRNAs in development have been uncovered, although these remain a relatively small fraction of all known miRNAs. What we have learnt so far suggests that, in addition to the molecular impact of a miRNA on its target/s, cell-type specificity and redundancy are important features that determine whether a miRNA has an evident function at the organismal level or not. Indeed, several redundancies have been uncovered. Some of the core miRNA families discussed here are composed of multiple, redundant members, typically expressed from multiple genomic loci. This is likely to allow these miRNAs to reach high concentrations in order to repress multiple targets. An extreme example may be *mir-430*, which in zebrafish contains 72 members, consistent with its ability to target a large number of maternal mRNAs (Giraldez et al., 2006). However, other very abundant miRNAs might have a stronger impact on one or a few targets: for example, a recent study in *C. elegans* showed that the very abundant *let-7* exerts its main function through a single target, *lin-41* (Ecsedi et al., 2015).

An interesting outcome of recent findings is that we may have in hand a small set of crucial core miRNAs that might account for the observed requirements for the miRNA biogenesis and effector machineries during embryonic development. However, it is also noticeable that although a lot of the focus has been placed on miRNAs acting in core processes, we know a lot less about miRNAs that could be acting at the periphery. This is probably due to the fact that the loss of very restricted miRNAs is unlikely to cause the types of broad defects that are typically looked for in broad reverse genetic screens (Chen et al., 2014; Miska et al., 2007). In addition, abundance is typically prioritized when deciding which candidate miRNA to follow up on. This is justifiably so, as miRNAs need to achieve a certain concentration within a cell to have a significant effect on its targets (Ameres and Zamore, 2013). However, it should be noted that when profiling complex cell mixtures, a low abundance read-out could be a result of high expression in a restricted cell type.

The evolution of miRNAs could hold a clue as to why so many of them may be expressed with high specificity. It has been proposed that most new miRNAs arise *de novo*, and that newly evolved miRNAs are more likely to cause detrimental regulation when they first appear. These miRNAs therefore can be tolerated if they are expressed at a low level and/or expressed in a cell type-specific manner (Chen and Rajewsky, 2007). How many of them have acquired a function then remains to be seen, but it is clear that several of these specific miRNAs provide functions in cellular diversification and specialization. Therefore, understanding the contribution of miRNAs to the generation of new cell types during development and evolution requires further exploration of this broad class of peripheral miRNAs.

Transcription factors (TFs) can also be considered in light of this hierarchy. Like miRNAs, many TFs occupy core positions and play essential roles in patterning the embryo and defining its basic body parts, whereas many others are known to have specific functions at the periphery (e.g. the many terminal selectors of neuronal identity described by Hobert, 2016). Yet it has been highlighted that, for example in *C. elegans*, although only ∼10% of miRNA deletions cause broad defects, loss of function of ∼30% of TFs do so (Hobert, 2008). One potential explanation for this could be that more miRNAs than TFs are expressed in highly restricted manner. Indeed, although it looks like a large fraction of miRNAs could fall into this 'highly restricted' category, a systematic study in *Drosophila* embryos shows that out of ∼700 TFs only 20% show 'single-organ specificity', and the majority are expressed across multiple organ systems (Hammonds et al., 2013). However, more systematic expression analyses allowing comparisons within species are needed to address this issue further. Although it is clear that the restriction of expression and function of miRNAs can be remarkable in animals like *C. elegans*, it is not clear whether this level of restriction occurs in other organisms. Grasping the extent of this specificity in animals with more complex tissues, both in terms of numbers of restricted miRNAs but also degree of specificity, will require approaches that provide single-cell resolution. The new era of single-cell sequencing will certainly provide interesting insights

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into this and will no doubt further aid our dissection of miRNA function in animal development.

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Competing interests

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